

## THE OCCURRENCE OF BRASSICASTEROL AND EPIBRASSICASTEROL IN THE CHROMOPHYCOTA

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**Abstract**—1. Sterols were identified from eight isolates of five species in the Chromophycota that were cultured axenically and harvested in the stationary phase.

2. Analyses were performed on four strains from the Prymnesiophyceae, two strains from the Cryptophyceae and one from the Bacillariophyceae. Most strains examined contained only one major sterol, 24-methyl-22-dehydrocholesterol.

3. Analysis by capillary GC, HPLC, and in one instance NMR, showed that the two strains provisionally identified as *Isochrysis* contained brassicasterol (24 $\beta$ -methyl-22-dehydrocholesterol); whereas, all other species examined contained primarily epibrassicasterol (24 $\alpha$ -methyl-22-dehydrocholesterol).

4. Stigmasterol (24 $\alpha$ -ethyl-22-dehydrocholesterol) accompanied epibrassicasterol in *Pleurochrysis carterae*.

5. Analyses of C-24 alkyl isomers in these algae may provide useful information concerning their taxonomic placement.

6. The occurrence of both isomers of 24-methyl-22-dehydrocholesterol in oysters is explained by the occurrence of both isomers among algae which are probably dietary sources for oysters.

### INTRODUCTION

Oysters, scallops and other marine invertebrates contain a wide array of sterols while lacking or possessing limited sterol biosynthetic ability. The major sterols of oysters and scallops are cholesterol, 24-methylenecholesterol, and 24-methyl-22-dehydrocholesterol (Teshima *et al.*, 1980; Patterson *et al.*, 1975). The latter sterol has been shown to be an isomeric mixture of brassicasterol (24 $\beta$ -methyl) and epibrassicasterol (24 $\alpha$ -methyl) in the oyster (Patterson, unpublished) and in the scallop (Khalil *et al.*, 1980). Although "brassicasterol" has been isolated from several protists which are possible oyster food sources, this sterol has not frequently been identified with respect to its C-24 orientation. *Isochrysis galbana* has been shown to contain 24-methyl-22-dehydrocholesterol in several studies (Volkman *et al.*, 1981; Marlow *et al.*, 1984; Lin *et al.*, 1982) but only Goad *et al.* (1983) determined that the sterol was 24 $\alpha$ -methyl-22-dehydrocholesterol (epibrassicasterol). Epibrassicasterol has also identified as the principal sterol of the Prymnesiophytes *Chrysotila lamellosa* (Raederstorff and Rohmer, 1984) and *Emiliana huxleyi* (Maxwell *et al.*, 1980), whereas the major sterol of *Hymenomonas carterae*, *H. pringsheimii*, and *Coccolithus pelagicus* was only identified as 24-methyl-22-dehydrocholesterol (Volkman *et al.*, 1981; Marlow *et al.*, 1984; Goad *et al.*, 1983). In the Cryptophyceae, *Cryptomonas* sp. was shown to produce epibrassicasterol; however, the sterol of *Chroomonas salina* was identified only

as 24-methyl-22-dehydrocholesterol (Goad *et al.*, 1983). A great many diatoms (especially the pennate diatoms) contain 24-methyl-22-dehydrocholesterol (Volkman, 1986), but only in *Phaeodactylum tricornutum* has it been specifically identified as epibrassicasterol (Rubinstein and Goad, 1974). Brassicasterol has been identified specifically in only one alga, an unidentified species of the order Sarcinochrysidales (Chrysophyceae) (Rohmer *et al.*, 1980; Kokke *et al.*, 1984). With  $\alpha/\beta$  mixtures of 24-methyl-22-dehydrocholesterol occurring in marine invertebrates, it would appear that other phytoplankton must be producing the 24 $\beta$  epimer or else some explanation is needed for the presence of this sterol in oysters and scallops. During a screening of algae for sterol composition in relation to their possible use in the feeding of oysters, we found nine strains of phytoplankton which contain 24-methyl-22-dehydrocholesterol as their principal sterol. The objective of this work was to determine the stereochemistry of these sterols at C-24.

### MATERIALS AND METHODS

#### Growth methods

Algal cultures utilized in this study were obtained from the Milford algal collection where they had been maintained for many years in enriched natural seawater medium, "E" formulation (Ukeles, 1973). At Milford, axenic test tube cultures were increased in volume through a series of progressively larger flasks and finally inoculated into carboy assemblies (Ukeles, 1973). Carboy cultures were operated semi-continuously, with harvests of about  $\frac{1}{3}$  of the total

culture volume (6 l taken from 18 l) on Friday of each week, followed immediately by replacement of harvested volumes with autoclave-sterilized "E" medium. Harvests and additions of growth medium were accomplished with sterile technique so that cultures remained axenic. Direct observation with the fluorescence microscope using acridine orange confirmed that cell suspensions subjected to sterol analysis were, indeed, axenic.

Algae for sterol analysis were harvested in the stationary phase of the growth cycle. Harvested cultures were concentrated by cold centrifugation (1020 g at 10°C for 20 min), and resuspended in a minimal volume of isotonic NaCl. This concentrated algal cell suspension was volumetrically aliquotted into glass ampoules, with a small volume being retained for microscopic counting in an Improved Neubauer hemacytometer. Ampoules containing algal cell suspensions were then frozen in a rotating bath of cold methanol (−25°C) and lyophilized using a VirTis (use of trade names does not imply endorsement) Unitra 10–100 manifold-style freeze-dryer. Ampoule necks were melt-sealed immediately upon removal from the lyophilizer manifold, and algal samples were stored in the sealed ampoules until analyzed.

#### Isolation and identification of sterols

Dry algal samples were extracted in chloroform/methanol (2:1) for 2–8 hr, in a Soxhlet apparatus. After removal of solvent, the lipid was saponified for 1 hr using 7% KOH in 70% aqueous methanol. The nonsaponifiable lipids were partitioned into diethyl ether, the solvent was removed after N<sub>2</sub>, and the nonsaponifiables were dissolved in hexane for alumina (Grade II) chromatography. Elution of fractions was accomplished with hexane, hexane/benzene (1:1), benzene, and diethyl ether, respectively. Sterols eluted in the ether fraction and were analyzed on a 15 m × 0.25 mm i.d. capillary SPB-1 column at 255°C in a Varian Model 3700 gas chromatograph interfaced with a Varian Model 401 Chromatographic Data System. Sterols were tentatively identified by retention times relative to cholesterol, and confirmation was obtained by GC–mass spectrometry on a Finnigan-MAT model 4512 gas chromatograph–mass spectrometer equipped with a 30 m × 0.32 mm i.d. fused silica capillary column with a 0.25 µm film of DB-1 (J & W Scientific). HPLC separation of sterol C-24 epimers was performed on a TSK-Gel ODS 120A column, 4.6 mm i.d. × 25 cm, 5 µm particle size (Tokyo Soda, Tokyo), as initially described by Ikekawa *et al.* (1989) for separation of steryl benzoates; however, a highly modified procedure was used. Free sterols were separated at 12°C by elution with methanol:isopropanol 4:1 at a flow rate of 1.0 ml/min controlled by a Spectra-Physics SP.8700XR solvent delivery system. Absorbance was monitored at 214 nm with a Waters model 441 detector connected to a Shimadzu Model C-R3A recording integrator. Each unknown sample was injected alone and with a cholesterol internal standard for accurate RRT determination. All HPLC peaks were trapped and analyzed by GLC. Because one unknown appeared to contain stigmasterol and/or poriferasterol, it was analyzed with a solvent of 100% methanol to facilitate resolution of the two epimers.

#### RESULTS

Each of the algal strains examined contained 24-methyl-22-dehydrocholesterol as the principal sterol, and in all strains except *Pleurochrysis carterae*, it was the only sterol identified. *Pleurochrysis carterae* contained two other sterols, each composing approximately 30% of the total sterol. The first of these was identified as 24-ethyl-22-dehydrocholesterol by its GLC-RRT of 1.36 and its mass spectrum showing a molecular ion at *m/z* 412 and other prominent peaks

at *m/z* 394, 379, 369, 351, 300, 271 and 255. Its apparent HPLC-RRT with 100% methanol was 0.84, but this value was inaccurate because the compound co-eluted with 24-methyl-22-dehydrocholesterol of this species during HPLC. The HPLC-RRTs of authentic standards of stigmasterol (24 $\alpha$ -ethyl-22-dehydrocholesterol) and poriferasterol (24 $\beta$ -ethyl-22-dehydrocholesterol) were 0.83 and 0.86, respectively. Identification of the unknown compound from *P. carterae* as stigmasterol was achieved by coinjection of the unknown compound with stigmasterol or poriferasterol during HPLC. Coinjection with stigmasterol resulted in detection of only one peak; two peaks were seen when the unknown was coinjected with poriferasterol. The remaining sterol in *P. carterae* was 23,24-dimethyl-22-dehydrocholesterol, a rare sterol previously found in *Pleurochrysis carterae* (as *Hymenomonas carterae*) (Volkmann, 1981), and in *Chattonella japonica* (Nichols *et al.*, 1983). The side chain stereochemistry of the latter sterol has not been determined.

The chromatographic characteristics of the 24-methyl-22-dehydrocholesterol isolated from the algal strains are compared to those of authentic brassicasterol and epibrassicasterol in Table 1. Gas chromatography with a 100 m column can separate the isomers of 24-methyl-22-dehydrocholesterol (Thompson *et al.*, 1981), and pure  $\alpha$  and  $\beta$  isomers can be distinguished from each other on a shorter column (Itoh *et al.*, 1981, 1982). In Table 1, GC analysis on a 15 m capillary column shows six strains with 24-methyl-22-dehydrocholesterol having GC characteristics matching those of epibrassicasterol and two match those of brassicasterol. Epibrassicasterol elutes from GC before brassicasterol in agreement with Thompson *et al.* (1981) and Itoh *et al.* (1982). When the above compounds were subjected to the reversed phase HPLC system which separates C-24 epimers (see Materials and Methods), the results were in complete agreement with the GC data (Table 1). Our data show epibrassicasterol (24 $\alpha$ ) being eluted before

Table 1. Chromatographic characteristics of 24-methyl-22-dehydrocholesterol isolated from algae compared with those of authentic C-24 epimers

Algal Species	Clone	GC-RRT*	HPLC-RRT
Cryptophyceae			
<i>Rhodomonas</i> sp.	Rhodo	1.097	0.65
<i>Rhodomonas lens</i>	†	1.098	0.65
Prymnesiophyceae			
<i>Pleurochrysis carterae</i>	Cocco II	1.098	0.64 (84%) 0.85 (16%)
<i>Dicrateria inornata</i>	Dicrat	1.097	0.64
<i>Isochrysis</i> sp.	(T-ISO)	1.101	0.85
<i>Isochrysis</i> sp.	(C-ISO)	1.103	0.84
Bacillariophyceae			
<i>Nitzschia closterium</i> (–)†	D-828	1.098	0.64
<i>N. closterium</i> (+)	D-828	1.097	0.64
Brassicasterol (synthesized from ergosterol)		1.102	0.85
Epibrassicasterol (isolated from <i>Phaeodactylum</i> )		1.098	0.64

\*GC and HPLC relative retention times are expressed relative to cholesterol = 1.000. SD of GC-RRT values was  $\pm 0.001$ .

†*Rhodomonas lens* was obtained from Dr C. Langdon of the University of Delaware. Other strain designations are those assigned in the Milford Microalgae Culture Collection. *Nitzschia closterium* was sampled from a silicon-deficient (–) and a silicon-sufficient (+) medium.

brassicasterol on HPLC, in agreement with Ikekawa *et al.* (1989). The HPLC data, however, have the additional advantage of greater resolution between these epimers than with GC so that mixed isomers can be detected, collected, and analyzed. By HPLC the 24-methyl-22-dehydrocholesterol in *P. carterae* contained 84% epibrassicasterol and 16% brassicasterol. The 24-methyl-22-dehydrocholesterol from all other algae examined was either pure brassicasterol or pure epibrassicasterol. The 24-methyl-22-dehydrocholesterol from *Rhodomonas lens* and *Isochrysis* sp. (T-ISO) initially appeared to contain small amounts of the opposite C-24 epimer i.e. 2% brassicasterol in *R. lens* and 0.5% epibrassicasterol in *Isochrysis*, respectively. Trapping of these minor peaks during HPLC and analysis of them by GLC indicated that they were not brassicasterol or epibrassicasterol. In addition to the above data, the epibrassicasterol from *Rhodomonas* sp. was further purified by digitonin precipitation, followed by recrystallization from methanol and analysis by  $^1\text{H}$  NMR. The NMR spectrum of the *Rhodomonas* sp. sterol was identical to that of epibrassicasterol, and differed from that of brassicasterol by the C-21 doublet being shifted slightly upfield in epibrassicasterol (Rubinstein and Goad, 1974; Chiu and Patterson, 1981; Khalil *et al.*, 1980).

#### DISCUSSION

The identification of epibrassicasterol in *Rhodomonas* sp. and *Rhodomonas lens* is in accord with the earlier demonstration of its presence in another cryptophyte, *Cryptomonas* sp. (Goad *et al.*, 1983). The only other cryptophyte examined for sterols (*Chroomonas salina*), also contained 24-methyl-22-dehydro-cholesterol (Goad *et al.*, 1983).

The sterol of *Nitzschia closterium* appears to be epibrassicasterol, and not brassicasterol as was earlier reported (Kanazawa *et al.*, 1971; Orcutt and Patterson, 1975). The earlier reports of brassicasterol were made without the benefit of modern methods which will distinguish between the two epimers. In light of these data, it would seem reasonable to regard other identifications of "brassicasterol" in the Nitzshiaceae (*N. frustulum*, and *N. ovalis*; Orcutt and Patterson, 1975) and *Stauroneis amphioxys*; Gillian *et al.*, 1981) as epibrassicasterol until an absolute determination is made.

The identification of epibrassicasterol in *Dicrateria inornata* and *Pleurochrysis carterae* (*Hymenomonas carterae*) is in accord with the composition of the closely related *Emiliania huxleyi* (Maxwell *et al.*, 1980). The identification of brassicasterol in two studies of *Isochrysis* (family Isochrysidaceae) runs counter to a previous report of epibrassicasterol in *Isochrysis galbana* (Goad *et al.*, 1983) and in *Chrysotila lamellosa* (Maxwell *et al.*, 1980), a species from the same family. Epibrassicasterol was identified from *Isochrysis* (Goad *et al.*, 1983) and from *Emiliania* (Maxwell *et al.*, 1980) by convincing NMR data. Although sufficient material was not available in this work for NMR analysis, HPLC separation of these isomers is so efficient that physical separation can be achieved.

The actual taxonomic relationships between the two strains, referred to in common practice as members of the genus *Isochrysis*, is largely unknown. The strain with the clone designation "ISO", (identical to the Plymouth Collection's strain PLY-1) is the type strain of the species *I. galbana* Parke, and its identity is therefore certain. By contrast, inclusion of the two other strains reported here, *Isochrysis* sp. "T-ISO" and "C-ISO", in the genus *Isochrysis* is essentially by convenience and unsupported by systematic convention. Unpublished results (Patterson) of sterol analysis of the "ISO" type strain show that *I. galbana* produces a more complex assortment of sterols than the two "*Isochrysis*" strains reported here. Besides gross morphological similarity, the sterol information presented here is the most convincing evidence that the two "*Isochrysis*" strains may be closely related to each other, although not necessarily included in the genus. However, a wider survey of the Haptophyceae will be required to determine if sterol characteristics, such as those reported here, are sufficiently distinctive to be useful at the generic and specific systematic levels.

The presence of brassicasterol or epibrassicasterol may be a significant characteristic for the taxonomy of these important, yet poorly understood organisms. Further research is needed to resolve these questions.

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#### REFERENCES

- Chiu P. L. and Patterson G. W. (1981) Quantitative estimation of C-24 epimeric sterol mixtures by 220-MHz nuclear magnetic resonance spectroscopy. *Lipids* **15**, 203–206.
- Gillian F. T., McFadden G. I., Wetherbee R. and Johns R. B. (1981) Sterols and fatty acids of an antarctic sea ice diatom *Stauroneis amphioxys*. *Phytochemistry* **20**, 1935–1937.
- Goad L. J., Holz G. G., Jr and Beach D. H. (1983) Identification of (24S)-24-methylcholesta-5,22-dien-3 $\beta$ -ol as the major sterol of a marine cryptophyte and a marine prymnesiophyte. *Phytochemistry* **22**, 475–476.
- Ikekawa N., Fujimoto Y., Kadota S. and Kikuchi T. (1989) Effective separation of sterol C-24 epimers. *J. Chromat.* **468**, 91–98.
- Itoh T., Fukushima K., Tamura T. and Matsumoto T. (1981) Gas chromatographic differentiation of C-24 epimeric alkylsterols on glass capillary column. *Yukagaku* **30**, 586–587.
- Itoh T., Tani H., Fukushima K., Tamura T. and Matsumoto T. (1982) Structure-retention time relationship of sterols and triterpene alcohols in gas chromatography on a glass capillary column. *J. Chromat.* **234**, 65–76.
- Kanazawa A., Yoshioka M. and Teshima S. (1971) The occurrence of brassicasterol in the diatoms, *Cyclotella nana* and *Nitzschia closterium*. *Bull. Jap. Soc. scient. Fish.* **37**, 899–903.
- Khalil M. W., Idler D. R. and Patterson G. W. (1980) Sterols of Scallop III. Characterization of some C-24 epimeric sterols by high resolution (220 MHz) nuclear magnetic resonance spectroscopy. *Lipids* **15**, 69–73.

- Kokke W. C. M. C., Shoolery J. N., Fenical W. and Djerassi C. (1984) Biosynthetic studies of marine lipids. 4. Mechanism of side chain alkylation in (E)-24-propylidenecholesterol by a chrysophyte alga. *J. org. Chem.* **49**, 3742–3752.
- Lin D. S., Idias A. M., Conner W. E., Caldwell R. S., Cory H. T. and Davies G. D. (1982) Composition and biosynthesis of sterols in selected marine phytoplankton. *Lipids* **17**, 818–824.
- Marlow I. T., Green J. C., Neal A. C., Brassel S. C., Eglington G. and Course P. A. (1984) Long chain (*n*-C37–C39) alkenones in the Prymnesiophyceae. Distribution of Alkenones and other lipids and their taxonomic significance. *Br. Phycol. J.* **19**, 203–216.
- Maxwell J. R., MacKenzie A. S. and Volkman J. K. (1980) Configuration at C-24 in steranes and sterols. *Nature* **286**, 694–697.
- Nichols P. D., Volkman J. K. and Johns R. B. (1983) Sterols and fatty acids of the marine unicellular alga, FCRG 51. *Phytochemistry* **22**, 1447–1452.
- Orcutt D. M. and Patterson G. W. (1975) Sterol, fatty acid and elemental composition of diatoms grown in chemically defined media. *Comp. Biochem. Physiol.* **50B**, 579–583.
- Patterson G. W., Khalil M. W. and Idler D. R. (1975) Sterols of scallop I. Application of hydrophobic Sephadex derivatives to the resolution of a complex mixture of marine sterols. *J. Chromat.* **115**, 153–159.
- Raedersdorff D. and Rohmer M. (1984) Sterols of the unicellular algae *Nematochryopsis roscoffensis* and *Chrysotila lamellosa*: isolation of (24E)-24*n*-propylidenecholesterol and 24-*n*-propylcholesterol. *Phytochemistry* **23**, 2835–2838.
- Rohmer M., Kokke W. C. M. C., Fenical W. and Djerassi C. (1980) Isolation of two new C30 sterols, (24E)-24-*N*-propylidenecholesterol and 24-*N*-propylcholesterol, from a cultured marine chrysophyte. *Steroids* **35**, 219–231.
- Rubinstein I. and Goad L. J. (1974) Occurrence of (24S)-24-methylcholesta-5,22-dienol in the diatom *Phaeodactylum tricornutum*. *Phytochemistry* **13**, 485–487.
- Teshima S., Patterson G. W. and Dutky S. R. (1980) Sterols of the oyster, *Crassostrea virginica*. *Lipids* **15**, 1004–1011.
- Thompson R. H., Jr, Patterson G. W., Thompson M. J. and Slover H. F. (1981) Separation of C-24 epimeric sterols by glass capillary gas liquid chromatography. *Lipids* **16**, 694–699.
- Ukeles R. (1973) *Handbook of Phycological Methods—Culture Methods and Growth Measurements*. (Edited by Stein J.), pp. 233–254. Cambridge University Press, London.
- Volkman J. K., Smith D. J., Eglington G., Forsberg T. E. V. and Corner E. D. S. (1981) Sterol and fatty acid composition of four marine haptophycean algae. *J. mar. Biol. Assoc. UK* **61**, 509–527.
- Volkman J. K. (1986) A review of sterol markers for marine and terrigenous organic matter. *Org. Geochem.* **9**, 83–89.